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(54) Title: PRODUCTION OF 1,3-PROPANEDIOL FROM GLYCEROL BY RECOMBINANT BACTERIA EXPRESSING RECOMBINANT DIOL DEHYDRATASE

(57) Abstract

A process is provided for the bioconversion of glycerol to 1,3-propanediol in which genes from a bacteria known to possess a diol dehydratase enzyme for 1,2-propanediol degradation are cloned into a bacterial host and the host is grown in the presence of glycerol; expression of the foreign genes in the host cell facilitates the enzymatic conversion of glycerol to 1,3-propanediol which is isolated from the culture.

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TITLE

PRODUCTION OF 1,3-PROPANEDIOL FROM GLYCEROL BY RECOMBINANT BACTERIA EXPRESSING RECOMBINANT DIOL DEHYDRATASE

FIELD OF INVENTION

This invention relates to a process for the bioconversion of glycerol to 1,3-propanediol by recombinant bacteria harboring a foreign gene encoding a diol dehydratase.

BACKGROUND

1,3-Propanediol is a monomer having potential utility in the production of polyester fibers and the manufacture of polyurethanes and cyclic compounds.

A variety of chemical routes to 1,3-propanediol are known. For example, 1,3-propanediol may be prepared from ethylene oxide and a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen and an acid; by the catalytic solution phase hydration of acrolein followed by reduction; or from hydrocarbons such as glycerol, reacted in the presence of carbon monoxide and hydrogen over periodic table group VIII catalysts. Although it is possible to generate 1,3-propanediol by these methods, they are expensive and generate waste streams containing environmental pollutants.

Biological routes to 1,3-propanediol are known which utilize feedstocks produced from renewable resources. For example, bacterial strains able to convert glycerol into 1,3-propanediol are found e.g., in the species *Klebsiella*, *Citrobacter*, *Clostridium*, and *Lactobacillus*. In these bacteria, glycerol can enter either an oxidative or reductive pathway. Oxidation of glycerol results in the conversion of glycerol to dihydroxyacetone (DHA) by glycerol dehydrogenase and the DHA is phosphorylated by an adenosine triphosphate (ATP) dependent kinase to yield dihydroxyacetone phosphate (DHAP) which enters the glycolytic pathway in the cell. Reduction of glycerol

is accomplished by an initial isomerization and dehydration catalyzed by glycerol dehydratase to yield 3-hydroxypropionaldehyde which is further reduced by 1,3-propanediol:NAD^+ oxidoreductase to yield 1,3-propanediol, a dead end cellular metabolite. The expression of at least the first two enzymes involved in the oxidative pathway as well as the two enzymes involved in the reductive pathway in *K. pneumoniae* are coordinately regulated. The four enzyme system is functionally linked where the production of 1,3-propanediol from glycerol is dependent on the presence of the reductants supplied by the DHA to DHAP pathway.

The genes responsible for the conversion of glycerol to 1,3-propanediol have been isolated and are all encompassed by the *dha* regulon. In order to make use of the potential advantages of higher protein expression and growth rate of recombinant bacteria, several attempts have been made to express the *dha* regulon as heterologous genes in *E. coli*. For example, the *dha* regulon from *Citrobacter* (Daniel et al., *FEMS Microbiol. Lett.*, 100, 281, (1992)) and *Klebsiella* (Tong et al., *Appl. Environ. Microbiol.*, 57, 3541, (1991); have been expressed in *E. coli* and have been shown to convert glycerol to 1,3-propanediol. The expression of the *dha* regulon in recombinant bacteria offers potential advantages over wild type production of 1,3-propanediol. The genes involved in the *dha* regulon provide both the enzymes and the necessary reductants needed for the efficient conversion of glycerol to 1,3-propanediol. However, simultaneous overexpression of both glycerol dehydrogenase and glycerol dehydratase results in some of the glycerol being converted to DHA. It would be advantageous to convert all the glycerol to 1,3-propanediol by expressing only the reductive pathway enzymes while providing a different substrate for the generation of reductant. A preferred system

would provide for a more efficient use of the glycerol substrate while maintaining high yields of diol product.

It has long been known that a number of bacteria are capable of using 1,2-propanediol as a sole carbon source. It is thought that this ability is conferred by a specific vitamin B_{12} dependent diol dehydratase which is encoded by the *pdu* operon. The *pdu* operon is linked to the *cob* operon which encodes enzymes needed for the biosynthesis of vitamin B_{12} and both operons are subject to the regulation of the same activator protein encoded by the *c pocR* gene.

Recently the genes encoding the diol dehydratase of *Klebsiella oxytoca* were cloned and sequenced and the genes were expressed in *E. coli*. Although active diol dehydratase was observed in these transformants, there is no evidence that these clones are able to metabolize a carbon substrate to 1,3-propanediol.

Various *Salmonella* and *Klebsiella* sp. are known to produce a diol dehydratase which catalyzes the conversion of 1,2-propanediol, under anaerobic conditions, to propionaldehyde and eventually to 1-propanol and propionic acid. The diol dehydratase has also been identified in *Clostridia*, and *Propionibacterium* but not in *E. coli*. The diol dehydratase from *Klebsiella* sp. can convert glycerol to 1,3-propanediol (Forage et al., Bacteriol, 149, 413 (1981)).

Although the primary function of the *pdu* diol dehydratase is in the metabolism of 1,2-propanediol, applicants have discovered that the expression of *K. pneumoniae* diol dehydratase in *E. coli* will catalyze the conversion of glycerol to 1,3-propanediol. The recombinant bacteria expressing the diol dehydratase pathway converts glycerol to the desired 1,3-propanediol product and is not dependent on a linked system as with the glycerol dehydratase system. Applicants have discovered that

transformation of recombinant bacteria with the *pdu* diol dehydratase genes from *Klebsiella* sp. affords a new, efficient and cost effective biological route to 1,3-propanediol.

SUMMARY OF THE INVENTION

The present invention comprises a cosmid comprising a DNA fragment of about 35 kb isolated from *Klebsiella pneumoniae* wherein said fragment encodes an active diol dehydratase enzyme having the restriction digest in Figure 5, columns numbered 4.

The present invention further comprises a transformed microorganism comprising a host microorganism and the above-described cosmid.

The present invention further comprises a gene encoding an active diol dehydratase enzyme having the DNA sequence as listed in SEQ ID NO.:1 or a gene encoding an active alcohol dehydrogenase having the DNA sequence as listed in SEQ ID NO.:2.

The present invention further comprises a transformed microorganism comprising a host microorganism and either of the above-described genes.

The present invention further comprises the bioconversion of a carbon substrate by transforming a microbial host with genes capable of expressing a diol dehydratase and contacting said transformed host with said substrate.

The present invention further comprises the bioconversion of a carbon substrate by transforming a microbial host with genes derived from a cosmid comprising a fragment of about 35 kb isolated from *Klebsiella pneumoniae* wherein said genes encode an active diol dehydratase enzyme and any other functional bacterial protein encoded by said cosmid, and contacting said transformed host with said substrate.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the gene organization of *pdu-cob* region of *K. pneumoniae*.

The DNA sequence was analyzed using the GCG-Wisconsin package and the open reading frames were compared to the *S. typhimurium* sequence using GAP. The percent identity and similarity are shown.

Figure 2 is a comparison of the amino acid sequence encoded by the *pduC* gene of *S. typhimurium* with the amino acid sequence encoded by the *pduC* gene of *K. pneumoniae*.

Figure 3 is a comparison of amino acid sequence coded by *pduC* gene of *K. pneumoniae* with that of glycerol dehydratase from *Citrobacter freundii* showing percent similarity and percent identity.

Figure 4 is a comparison of the amino acid sequence deduced from an open reading frame of the glycerol dehydratase gene from *K. pneumoniae* with the amino acid sequence encoded by the same gene from *Citrobacter freundii*. The figure shows the percent similarity and percent identity between the two deduced amino acid sequences.

Figure 5 depicts restriction digests (EcoR 1, BamH 1, EcoR V and Not1) of cosmids pKP1, pKP2 and pKP4, labeled as columns 1, 2 and 4 respectively, and separation on a 0.8% agarose gel electrophoresis. Molecular size markers were loaded on the lanes in the end. Columns labeled as number 4 represent the cosmid containing a diol dehydratase enzyme.

DETAILED DESCRIPTION OF THE INVENTION

As used herein the following terms may be used for interpretation of the claims and specification.

The term "construct" refers to a plasmid, virus, autonomously replicating sequence, phage or nucleotide sequence, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

The term "transformation" or "transfection" refers to the acquisition of new genes in a cell after the incorporation of nucleic acid.

The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

The term "plasmid" or "vector" or "cosmid" as used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules.

The term "carbon substrate" means any carbon source capable of being metabolized by a microorganism wherein the substrate contains at least one carbon atom.

The term "dehydratase enzyme" will refer to any enzyme that is capable of converting a glycerol molecule to the product 3-hydroxypropionaldehyde. For the purposes of the present invention the dehydratase enzymes are either a glycerol dehydratase or a diol dehydratase having preferred substrates of glycerol and 1,2-propanediol, respectively.

The term "1,3-propanediol" refers to a compound of the formula $HOCH_2-CH_2-CH_2OH$, useful as a monomer in the production of polymers for fiber manufacture.

The following strains were deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) (12301 Packlawn Drive, Rockville, MD 20852, USA): ATCC 69789 corresponds to *E. coli* DH5α containing cosmid pKP1. ATCC 69790 refers to *E. coli* DH5α containing cosmid pKP4.

The present invention comprises a process for a biological production of 1,3-propanediol from glycerol

using recombinant organisms. The process incorporates a transformed *E. coli* bacteria, transformed with a heterologous *pdu* diol dehydratase gene, having a specificity for 1,2-propanediol. The transformed *E. coli* is grown in the presence of glycerol as a carbon source and 1,3-propanediol is isolated from the growth media.

The process of the present invention provides a rapid, inexpensive and environmentally responsible source of 1,3-propanediol monomer useful in the production of polyesters and other polymers.

The invention provides a transformed host cell suitable for the expression of *pdu* diol dehydratase. Suitable host cells will generally be those that do not normally harbor a diol dehydratase gene. Preferred in the process of the present invention are *E. coli*, *Bacillus subtilis*, *Bacillus licheniformis* or *Pichia pastoris*. The diol dehydratase within the transformed host cell has been previously described by Toraya et al., *J. Biol. Chem.*, 252, 963, (1977).

Isolation of Genes:

The *pdu* diol dehydratase gene is obtained from any suitable source, but preferably from a bacteria known to be able to use 1,2-propanediol as a sole carbon source. Suitable bacteria known to harbor the *pdu* gene include but are not limited to *Klebsiella* sp., *Clostridia* sp., *Salmonella* sp., and *Citrobacter* sp.

Methods of obtaining desired genes from a bacterial genome are common and well known in the art of molecular biology. In the present invention virtually any method may be used to isolate the gene encoding the desired diol dehydratase. For example, if the sequence of the gene is known, suitable genomic libraries created by restriction endonuclease digestion may be screened with probes complementary to the desired gene sequence. Once the sequence is isolated, the DNA may be amplified using standard

primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. 4,683,202) to obtain amounts of DNA suitable for transformation using appropriate vectors.

Alternatively cosmid libraries may be created where large segments of genomic DNA (35-45 kb) may be packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally cosmid vectors have at least one copy of the *cos* DNA sequence which is needed for packaging and subsequent circularization of the foreign DNA. In addition to the *cos* sequence these vectors will also contain an origin of replication such as ColE1 and drug resistance markers such as a gene resistant to ampicillin or neomycin. A number of cosmid vectors are known in the art such as pJB8 (Ish-Horowicz et al., *Nucl. Acids Res.* 9, 2989 (1981)), containing an *amp* marker, ColE1 origin of replication and a singel *cos* site; and, c2RB (Bates et al., *Gene*, 26, 137, (1983)), containing 2 *cos* sites, both kanamycin and ampicillin resistance genes and the ColE1 origin of replication. Although any cosmid vector is suitable for use in the present invention the vector Supercos 1 provided by Stratagene (La Jolla, CA) is most preferred.

Typically, to clone cosmids, foreign DNA is isolated and ligated, using the appropriate restriction endonucleases, adjacent to the *cos* region of the cosmid vector. Cosmid vectors containing the linearized foreign DNA is then packaged *in vitro* in DNA packaging vehicle such as bacteriophage λ. During the packaging process the *cos* sites are cleaved and the foreign DNA is packaged into the head portion of the bacterial viral particle. These particles are then used to transfect suitable host cells such as *E. coli*. Once injected into the cell, the foreign DNA circularizes under the influence of the *cos* sticky

ends. In this manner, large segments of foreign DNA can be introduced and expressed in recombinant host cells.

Cosmid vectors and cosmid transformation methods were used within the context of the present invention to clone large segments of genomic DNA from bacterial genera known to possess genes capable of processing glycerol to 1,3-propanediol. Specifically, genomic DNA from *K. pneumoniae* and *K. aerogenes* was isolated by methods well known in the art and digested with the restriction enzyme Sau3A for insertion into a cosmid vector Supercos 1 and packaged using GigapackII™ packaging extracts. Following construction of the vector *E. coli* XL1-Blue MR cells were transformed with the cosmid DNA. Transformants were screened for the ability to convert glycerol to 1,3-propanediol by growing the cells in the presence of glycerol and analyzing the media for 1,3-propanediol formation.

The DNA sequences generated from cosmid transformations named pKP4 and pKP5 were compared to DNA sequences in the Genbank data base. Several independent clones showing homology to *pdu* region of *S. typhimurium* were identified, suggesting that these transformants carried DNA encoding 1,2 propanediol utilizing enzymes including a 1,2-diol dehydratase gene. In contrast, in transformants named pKP1 and pKP2, an open reading frame showed extensive homology to the glycerol dehydratase gene from *C. freundii*, suggesting that these transformants containing DNA encoding the glycerol dehydratase gene.

Cells:

The present invention further comprises a transformed host cell capable of converting a carbon substrate to 1,3-propanediol. As disclosed above, host cells may be transformed with a single gene, encoding the diol dehydratase, a series of specific genes encoding the diol dehydratase and other enzymes known to facilitate the process of bioconversion or

with an entire cosmid DNA fragment. Preferred for use in the present invention is DH5α *E. coli*. However, it is contemplated that other cells will be amenable to transformation with the instant genes and will include, but are not limited to, other microorganisms such as *Bacillus* sp., *Klebsiella* sp., *Citrobacter* sp., *Clostridia* sp. and *Pichia* sp.

Carbon Substrate:

The present invention provides a carbon substrate which is converted to the desired 1,3-propanediol end product via the enzymatic machinery of the transformed host organism. Virtually any carbon substrate that will serve as a substrate for a dehydratase enzyme is suitable for the present invention where alcohols are of greatest use. Preferred carbon substrates will include, but are not limited to, glycerol, ethylene-glycol, 1,2-propanediol, 1,2-butanediol, and 2,3-butanediol, wherein glycerol is most preferred.

Purification and Isolation of 1,3-propanediol:

Methods for the purification of 1,3-propanediol from fermentation media are known in the art. For example propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (U.S. 5356812). A particularly good organic solvent for this process is cyclohexane (U.S. 5008473).

1,3-Propanediol may be identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. Preferred in the present invention is a method where fermentation media is analyzed on an analytical ion exclusion column using a mobile phase of 0.01 N sulfuric acid in an isocratic fashion.

The following Examples are meant to illustrate the invention but are not intended to limit it in any way.

EXAMPLES

GENERAL METHODS

Restriction enzyme digestions, phosphorylations, ligations and transformations were done as described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989). GeneClean (Stratagene, La Jolla, CA) was used to remove enzymes from restriction digests, as specified by the manufacturers. Restriction enzymes were obtained from New England Biolabs (Boston, MA) or Promega (Madison, WI) Growth media was obtained from GIBCO/BRL (Gaithersburg, MD)

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), and "d" means day(s).

Media:

Synthetic S12 medium was used in the screening of bacterial transformants for the ability to make 1,3-propanediol. S12 medium contains: ammonium sulfate, 10 mM; potassium phosphate buffer, pH 7.0, 50 mM; $MgCl_2$, 2 mM; $CaCl_2$, 0.7 mM; $MnCl_2$, 50 uM; $FeCl_3$, 1 uM; ZnCl, 1 uM; $CuSO_4$, 1.72 uM; $CoCl_2$, 2.53 uM; Na_2MoO_4, 2.42 uM; and thiamine hydrochloride, 2 uM.

Synthetic S15 medium was also used in the screening of bacterial transformants for the ability to make 1,3-propanediol. S15 medium contains: ammonium sulfate, 10 mM; potassium phosphate buffer, pH 7.0, 1 mM; MOPS/KOH buffer, pH 7.0, 50 mM; $MgCl_2$, 2 mM; $CaCl_2$, 0.7 mM; $MnCl_2$, 50 uM; $FeCl_3$, 1 uM; ZnCl, 1 uM; $CuSO_4$, 1.72 uM; $CoCl_2$, 2.53 uM; Na_2MoO_4, 2.42 uM; and thiamine hydrochloride, 2 uM.

Isolation and Identification 1,3-propanediol:

The conversion of glycerol to 1,3-propanediol was monitored by HPLC. Analyses were performed using a Waters Maxima 820 HPLC system using UV (210 nm) and RI detection. Samples were injected onto a Shodex SH-1011 column (8 mm x 300 mm, purchased from Waters,

Milford, MA) equipped with a Shodex SH-1011P precolumn (6 mm x 50 mm), temperature controlled at 50°C, using 0.01 N H_2SO_4 as moble phase at a flow rate of 0.5 mL/min. When quantitative analysis was desired, samples were prepared with a known amount of trimethylacetic acid as external standard. Typically, the retention times of glycerol (RI detection), 1,3-propanediol (RI detection), and trimethylacetic acid (UV and RI detection) were 20.67 min, 26.08 min, and 35.03 min, respectively.

Production of 1,3-propanediol was confirmed by gas chromatography/mass spectrometry (GC/MS) with a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5971 Series mass selective detector (EI) and a HP-INNOWax column (30 m length, 0.25 mm i.d., 0.25 micron film thickness). The retention time and mass spectrum of 1,3-propanediol generated from glycerol were compared to that of authentic 1,3-propanediol (*m/e*: 57, 58).

Cells:

Host cells used for cosmid transformations were *E. coli* DH5α fully described in Jesse et al., *Focus*, 10, 69 (1988) and obtained from GIBCO/BRL.

Construction of *K. pneumoniae* and *K. aerogenes* cosmid libraries:

K. pneumoniae (ATCC 25955) and *K. aerogenes* ((*K. pneumoniae* or *Aerobacter aerogenes*) ECL 2106) obtained from Dr. E.C.C. Lin, Harvard Medical School, Cambride, MA, and described in Ruch, F.E. and Lin, E.C.C., Journal of Bacteriology, Vol. 124, p. 348 (Oct. 1975), were grown in 100 ml LB medium for 8 h at 37°C with aeration. Bacteria (25 ml per tube) were centrifuged at 3,000 rpm for 15 min in a DuPont Sorvall GLC 2.B centrifuge at room temperature. The bacteria were pelleted and supernatant was decanted. The bacterial cell pellet was frozen at -20°C. The chromosomal DNA was isolated as outlined below with special care taken to avoid shearing of DNA (i.e.,

vortexing was avoided). One tube of bacteria was resuspended in 2.5 ml of 50 mM Tris-10 mM EDTA and 500 ul of lysozyme (1 mg/ml) was added. The pellet was gently resuspended and the suspension was incubated at 37°C for 15 min. Sodium dodecyl sulfate was added to bring the final concentration to 0.5%. This resulted in the solution becoming clear. Proteinase K (50 ug/ml) was added and the suspension was incubated at 55°C for 2 h. The tube was removed and transferred to an ice bath and sodium chloride was added to yield a 0.4 M final concentration. Two volumes of ethanol were added to the liquid. A glass tube was inserted to the interface and the DNA was gently spooled. DNA was dipped into a tube containing 70% ethanol. After drying in vacuo, the DNA was resuspended in 500 ul of water and the concentration of DNA was determined spectrophotometrically. A diluted aliquot of DNA was run on a 0.5% agarose gel to determine the intact nature of DNA.

The chromosomal DNA was partially digested with Sau3A as outlined by Sambrook et al., *supra*. DNA (2 ug) was digested with 2 units of Sau3A (Promega, Madison, WI) at room temperature in 200 ul of total volume. At 0, 5, 10 and 20 min, samples (50 ul) were removed and transferred to tubes containing 5 umol of EDTA. These tubes were incubated at 70°C for 10 min. An aliquot (2 ul) was withdrawn and analyzed on a 0.5% agarose gel electrophoresis to determine the level of digestion and the rest of the sample (48 ul) was stored at -20°C. The gel was stained with ethidium bromide and visualized under UV to determine the partial digestion of the chromosomal DNA. A decrease in the size of the chromosomal DNA with increase in time was observed showing that the decrease in the size of the chromosomal DNA is due to the action of Sau3A. DNA was extracted from rest of the sample by standard protocol methods (Sambrook et al., *supra*).

A cosmid library of partially digested DNA from *K. pneumoniae* or *K. aerogenes* was prepared using Supercos cosmid vector kit and GigapackII™ packaging extracts using reagents purchased from Stratagene (La Jolla, CA). The instructions provided by the manufacturer were followed. The packaged *K. pneumoniae* contained 4 x 10^4 to 1.0 x 10^5 phage titer and the packaged *K. aerogenes* contained 1.2 x 10^5 phage per mL as determined by transfecting *E. coli* XL1-Blue MR.

Cosmid DNA was isolated from 6 of the *E. coli* transformants and found to contain large insert of DNA (25 to 30 kb).

EXAMPLE 1

Screening *E. coli* strains, transformed with a cosmid library DNA from *K. pneumoniae* and containing the glycerol dehydratase enzyme that produce 1,3- propanediol

Example 1 demonstrated the screening of transformed *E. coli* cells with cosmid library DNA from *K. pneumoniae* for the presence of an enzyme that converted glycerol to 1,3-propanediol. Sequencing of two positive clones revealed that each contained a gene with a high degree of homology to the gene encoding glycerol dehydratase.

Six transformation plates containing approximately 1,000 colonies of *E. coli* XL1-Blue MR transfected with *K. pneumoniae* DNA were washed with 5 ml LB medium and centrifuged. The bacteria were pelleted and resuspended in 5 ml LB medium + glycerol. An aliquot (50 ul) was inoculated into a 15 ml tube containing S12 synthetic medium with 0.2% glycerol + 400 ng per ml of vitamin B_{12} + 0.001% yeast extract + 50 ug/ml ampicillin (50 amp). The tube was filled with the medium to the top, wrapped with parafilm and incubated at 30°C. A slight turbidity was observed after 48 h. Aliquots, analyzed for product distribution as described above at 78 h and 132 h,

were positive for 1,3-propanediol, the later time points containing increased amounts of 1,3-propanediol.

The bacteria, testing positive for 1,3-propanediol production were plated onto a LB + 50 amp, and serial dilutions were performed in order to isolate single colonies. Forty-eight single colonies were isolated and checked again for the production of 1,3-propanediol. Cosmid DNA was isolated from 6 independent clones and transformed into *E. coli* strain DH5α. The transformants were again checked for the production of 1,3-propanediol. Two transformants were characterized further and designated as DH5α-pKP1 and DH5α-pKP2.

DNA sequence analyses of DH5α-pKP1 and DH5α-pKP2 showed the presence of both glycerol dehydrogenase and glycerol dehydratase genes. Furthermore, the glycerol dehydratase gene of the transformed *E. coli* shared 96% similarity and 95% identity to the glycerol dehydratase gene from *Citrobacter freundii* (Figure 4). Thus, pKP 1 and 2 appeared to contain the *dha* regulon genes from *K. pneumoniae*.

EXAMPLE 2

Screening *E. coli* strains transformed with a cosmid library DNA from *K. pneumoniae* and containing the 1,2-propanediol dehydratase enzyme that produce 1,3-propanediol

Example 2 demonstrated the screening of *E. coli* cells, transformed with cosmid library DNA from *K. pneumoniae*, for the presence of an active enzyme that enabled the conversion of glycerol to 1,3-propanediol. Sequencing of the positive clones revealed that each contained a gene with a high degree of homology to the gene encoding 1,2-propanediol dehydratase, encoded by the *pdu* operon.

Single colonies of *E. coli* XL1-Blue MR transfected with packaged cosmid DNA from *K. pneumoniae* were inoculated into microtiter wells

containing 200 ul of S15 medium + 0.2% glycerol + 400 ng/ml of vitamin B_{12} + 0.001% yeast extract + 50 ug/ml ampicillin (50 amp). In addition to the microtiter wells, a master plate containing LB + 50 amp was also inoculated. After 96 h, 100 ul was withdrawn and centrifuged in a Rainin microfuge tube containing a 0.2 micron nylon membrane filter. Bacteria were retained and the filtrate was processed for HPLC analysis. Positive clones demonstrating 1,3-propanediol production were identified after screening approximately 240 colonies. Three positive clones were identified, two of which had grown on LB + 50 amp and one of which had not. Single colonies were isolated from the two positive clones grown on LB + 50 amp and verified for the production of 1,3-propanediol and designated as pKP4 and pKP5. Cosmid DNA was isolated from *E. coli* strains containing pKP4 and pKP5 and *E. coli* strain DH5α was transformed. Six independent transformants were verified for the production of 1,3-propanediol. *E. coli* strain DH5α containing pKP4 or pKP5 was able to convert glycerol to 1,3-propanediol as described below.

Production of 1,3-Propanediol with E. coli strains DH5α-pKP4 and DH5α-pKP5

A 2 mL screw capped cyrogenic vial, filled to capacity with media, was inoculated with *E. coli* strain DH5α containing pKP4 or pKP5 and incubated at 30°C. The media was composed of S12 medium supplemented with 0.01% yeast extract, 0.008% casamino acids, 50 ug/mL ampicillin, 10 ug/mL kanamycin, 0.4 ug/mL vitamin B_{12}, and either 0.2% glycerol or 0.1% glycerol plus 0.1% D-glucose. Inoculation was performed directly from an agar plate culture (LB supplemented with 50 ug/mL ampicillin). After 66 hr, growth was determined by the absorbance at 600 nm (OD_{600}) and the extent of reaction and product distribution determined by HPLC. The results are

presented in Table 1 and Table 2: the sample is identified by the transformant with a suffix notation indicating independent transformants, Gly is glycerol, Glu is D-glucose, Con. is conversion, Sel. is selectivity, Yld is yield, and NA is not applicable. Conversion, selectivity and yield were based on glycerol consumption.

TABLE 1

Production of 1,3-Propanediol from Glycerol

Sample	OD_{600}	[Gly] (mM)	[1,3-propanediol] (mM)	% Con.	% Sel.	% Yld.
media	NA	23.0	0.0	NA	NA	NA
pKP4-3	0.206	14.0	1.0	39	11	4
pKP4-4	0.297	12.6	1.6	45	15	7
pKP5-1	0.242	13.4	0.8	42	8	4
pKP5-2	0.300	13.4	1.4	42	15	6

TABLE 2

Production of 1,3-Propanediol from Glycerol and Glucose

Sample	OD_{600}	[Gly] (mM)	[Glu] (mM)	[1,3-propanediol] (mM)	% Con.	% Sel.	% Yld.
media	NA	10.7	4.3	0.0	NA	NA	NA
pKP4-3	0.257	5.3	0.0	1.0	50	19	9
pKP4-4	0.321	3.9	0.0	1.2	64	18	11
pKP5-1	0.366	1.5	0.2	3.6	86	39	34
pKP5-2	0.367	1.5	0.2	4.1	86	45	38

DNA sequence analysis of pKP4 and pKP5

The size of insert DNA in the case of both pKP4 and pKP5 varied from 25 to 30 Kb. Both clones had certain fragments that were common and certain fragments were different. A 22 kb EcoR1 fragment from pKP4 was eluted from an agarose gel using GeneClean and later digested with BamHI or EcoRV and the various fragments were subcloned into plasmid pIBI31 digested with EcoR1 or BamH1 or HinCII. Clones containing inserts were identified and DNA sequence was generated.

The DNA sequence that was generated showed homology to the *cob* and *pocR* and *pdu* genes of *S. typhimurium*. It is well known that the *pdu* operon in *S. typhimurium* codes for genes that are needed for 1,2-propanediol utilization. (Bobik et al., *J. Bacteriol*, 174, 2253 (1992)). Similarly, it is known that the *cob* operon encodes genes that are needed for vitamin B_{12} synthesis. Within the *pdu* operon it is further recognized that the *pduC* gene encodes for diol dehydratase production.

The region of *K. pneumoniae* coding for the *pdu* operon genes is shown in Figure 1. Figure 1 is a schematic representation of the gene organization of *pdu-cob* region of *K. pneumoniae*. Comparisons were made between this *pdu-cob* region and the same regions of the gene belonging to *S. typhimurium* using algorithms provided by Sequence Analysis Software of the University of Wisconsin (Genetics Computer Group, (1991), Version 7, April 1991, 575 Science Drive, Madison, WI, USA 53711). A table giving the percent identity and similarity as calculated by the GAP program of the Genetics Computer Group are shown below.

	Percent Similarity		Percent Identity
pocR	90.48%		84.35%
pduA	100%		94.85%
pduB	99.16%		96.64%
pduC	98.31%	(partial seq.)	94.92%
pduF	92.42%		82.20%

As can be seen by this comparison, and in Figure 2, the *pduC* open reading frame showed extensive homology (98.31%) to the *pduC* gene of *S. typhimurium*. *pduC* was linked to *pduF* and showed homology to the gene encoding glycerol dehydratase from *Citrobacter freundii* (Figure 3).

Figure 3 is a comparison of the deduced amino acid sequence encoded by the *pduC* gene from

K. pneumoniae vs. the amino acid sequence encoded by the glycerol dehydratase gene of *C. freundii*. These comparisons showed that the percent similarity was only 84% and the identity only 70%. Thus, the *pduC* gene encoding for diol dehydratase was a clearly different enzyme and is being used to convert glycerol to 1,3-propanediol in these transformed *E. coli* strains. The sequence of the gene encoding this diol dehydratase enzyme is given in SEQ ID NO.:1.

Additionally, another open reading frame has been identified on the *pdu* gene which showed a high degree of homology with the regions encoding alcohol dehydrogenases. For example, deduced amino acid comparisons showed that this open reading frame had 43% homology with *E. coli* alcohol dehydrogenase and a 54% homology with the oxidoreductase of *C. freundii*. This open reading frame had been sequenced and is identified as SEQ ID NO.:2.

EXAMPLE 3

Screening *E. coli* strains, transformed with a cosmid library DNA from *K. aerogenes* and containing the glycerol dehydratase enzyme that produce 1,3-propanediol.

Example 3 demonstrated the screening of transformed *E. coli* cells with cosmid library DNA from *K. aerogenes* for the presence of an active enzyme that converted glycerol to 1,3-propanediol. Sequencing of the positive clones revealed that each contained a gene with a high degree of homology to the gene encoding 1,2-propanediol dehydratase, endcoded by the *pdu* operon.

Single colonies of *E. coli* XL1-Blue MR transfected with DNA from *K. aerogenes* were inoculated into microtiter wells containing 200 ul of S15 medium + 0.2% glycerol + 400 ng per ml of vitamin B_{12} + 0.001% yeast extract + 50 ug/ml ampicillin (50 amp).

Culture supernatant was analyzed for the presence of 1,3-propanediol after 96 h. Two colonies were

positive from 2 microtiter plates but after 1 week at room temperature the bacteria were not viable. A third mirotiter plate was inoculated and a master plate containing LB + 50 amp was also inoculated. One positive clone labelled KAE3E10 was identified. The masterplate containing KAE3E10 used to replate the positive clone and cosmid DNA was isolated. DH5α cells were transformed with KAE3E10 DNA and transformants were screened for the conversion of glycerol to 1,3-propanediol. KAE3E10 was renamed pKA3 and contained an insert of approximately 40 kb. The DNA sequence of pKA3 showed a region that was homologous to *cob* and *pocR* and *pdu* operon of *S. typhimurium*.

Thus, it appeared that pKA3 also coded for a 1,2-propanediol utilizing operon. Diol dehydratase was presumably responsible for the conversion of glycerol to 1,3-propanediol.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE (ZIP): 19898
(G) TELEPHONE: 302-892-8112
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: PRODUCTION OF 1,3-PROPANEDIOL FROM GLYCEROL BY RECOMBINANT BACTERIA EXPRESSING RECOMBINANT DIOL DEHYDRATASE

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM
(C) OPERATING SYSTEM: MICROSOFT WINDOWS 3.1
(D) SOFTWARE: MICROSOFT WORD 2.0C

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/440,377
(B) FILING DATE: MAY 12, 1995

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: LINDA AXAMETHY FLOYD
(B) REGISTRATION NUMBER: 33,692
(C) REFERENCE/DOCKET NUMBER: CR-9692-A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4746 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAGATCGA AAAGATTTGA AGCACTGGCG AAACGCCCTG TGAATCAGGA TGGTTTCGTT 60

AAGGAGTGGA TTGAAGAGGG CTTTATCGCG ATGGAAAGTC CTAACGATCC CAAACCTTCT 120

ATCCGCATCG TCAACGGCGC GGTGACCGAA CTCGACGGTA AACCGGTTGA GCAGTTCGAC 180

CTGATTGACC ACTTTATCGC GCGCTACGGC ATTAATCTCG CCCGGGCCGA AGAAGTGATG 240

GCCATGGATT CGGTTAAGCT CGCCAACATG CTCTGCGACC CGAACGTTAA ACGCAGCGAC 300

ATCGTGCCGC TCACTACCGC GATGACCCCG GCGAAAATCG TGGAAGTGGT GTCGCATATG 360

AACGTGGTCG AGATGATGAT GGCGATGCAA AAAATGCGCG CCCGCCGCAC GCCGTCCCAG 420

CAGGCGCATG TCACTAATAT CAAAGATAAT CCGGTACAGA TTGCCGCCGA CGCCGCTGAA 480

GGCGCATGGC GCGGCTTTGA CGAACAGGAG ACCACCGTCG CCGTGGCGCG CTACGCGCCG 540

TTCAACGCCA TCGCCCTGCT GGTGGGTTCA CAGGTTGGCC GCCCCGGCGT CCTCACCCAG 600

TGTTCGCTGG AAGAAGCCAC CGAGCTGAAA CTGGGCATGC TGGGCCACAC CTGCTATGCC 660

GAAACCATTT CGGTATACGG TACGGAACCG GTGTTTACCG ATGGCGATGA CACTCCATGG 720

TCGAAAGGCT TCCTCGCCTC CTCCTACGCC TCGCGCGGCC TGAAAATGCG CTTTACCTCC 780

GGTTCCGGTT CTGAAGTACA GATGGGCTAT GCCGAAGGCA AATCGATGCT TTATCTCGAA 840

GCGCGCTGCA TCTACATCAC CAAAGCCGCC GGGGTGCAAG GCCTGCAGAA TGGCTCCGTC 900

AGCTGTATCG GCGTACCGTC CGCCGTGCCG TCCGGGATCC GCGCCGTACT GGCGGAAAAC 960

CTGATCTGCT CAGCGCTGGA TCTGGAGTGC GCCTCCAGCA ACGATCAAAC CTTTACCCAC 1020

TCGGATATGC GGCGTACCGC GCGTCTGCTG ATGCAGTTCC TGCCAGGCAC CGACTTCATC 1080

TCCTCCGGTT ACTCGGCGGT GCCCAACTAC GACAACATGT TCGCCGGTTC CAACGAAGAT 1140

GCCGAAGACT TCGATGACTA CAACGTGATC CAGCGCGACC TGAAGGTCGA TGGCGGCCTG 1200

CGGCCGGTGC GTGAAGAGGA CGTGATCGCC ATTCGCAACA AAGCCGCCCG CGCGCTGCAG 1260

GCGGTATTTG CCGGCATGGG TTTGCCGCCT ATTACGGATG AAGAGGTAGA AGCCGCCACC 1320

TACGCCCACG GTTCAAAAGA TATGCCTGAG CGCAATATCG TCGAGGACAT CAAGTTTGCT 1380

CAGGAGATCA TCAACAAGAA CCGCAACGGC CTGGAGGTGG TGAAAGCCCT GGCGAAAGGC 1440

GGCTTCCCCG ATGTCGCCCA GGACATGCTC AATATTCAGA AAGCCAAGCT CACCGGCGAC 1500

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ATTAAAAATA TCCCGGGCGC GCTCGATCCC AATGAACTTG GCTAAGGGGT GAAAAATGGA 1680

AATTAACGAA ACGCTGCTGC GCCAGATTAT CGAAGAGGTG CTGTCGGAGA TGAAATCAGG 1740

CGCAGATAAG CCGGTCTCCT TTAGCGCGTC TGCGGCTTCT GTCGCCTCTG CCGCGCCGGT 1800

CGCCGTTGCG CCTGTGTCCG GCGACAGCTT CCTGACGGAA ATCGGCGAAG CCAAACCCGG 1860

CACGCAGCAG GATGAAGTCA TTATTGCCGT CGGGCCAGCG TTTGGTCTGG CGCAAACCGC 1920

CAATATCGTC GGCATTCCGC ATAAAAATAT TCTGCGCGAA GTGATCGCCG GCATTGAGGA 1980

AGAAGGCATC AAAGCCCGGG TGATCCGCTG CTTTAAGTCA TCTGACGTCG CCTTCGTGGC 2040

AGTGGAAGGC AACCGCCTGA GCGGCTCCGG CATCTCGATC GGTATTCAGT CGAAAGGCAC 2100

CACCGTCATC CACCAGCGCG GCCTGCCGCC GCTTTCCAAT CTGGAACTCT TCCCGCAGGC 2160

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ATTCGCCACA GCGCGTTGGC TGAAACCACG GGTATAAAAG GCACATTACG AAATGTGTTC 3060

GGTATCCAGG AGGCGCTAAC GCAGGCGGCA AAAGCGGCCG GCATTCAGCT CAGCGATATT 3120

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AATGCGGCAA CGGCAGCGGG CTATCAGATA ACCGGCATTA TTTTGCAGCA GGATGACGGC 3420

GTGCTGGTCA ATAACCGGCT ACAGCAACCG CTACCGGTGA TCGACGAAGT TCAGCATATC 3480

GACCGGATTC CACTTGGCAT GCTGGCGGCC GTCGAGGTCG CTTTACCCGG TAAGATCATC 3540

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ACCCCCTCCG GCGACGTCAA GGCCCGCGCT ATTCCGGCAG GTAATCTGTT GCTCATCGCT 3720

CAGGGGCGCA GCGTACAGGT TGATGTGGCC GCCGGGGCGG AAGCCATCAT GAAAGCGGTT 3780

GACGGCTGCG GCAAACTGGA CAACGTCGCG GGAGAAGCGG GCACCAATAT CGGCGGCATG 3840

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GACGCGCTGG CGCACTACCG GCTGGTTGCC GGGCGCGGCA ACATCCGCGG CTGTGAAGGC 4680

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GAGTAG 4746

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1335 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGCATACCT TTTCTCTGCA AACGCGCCTC TACAGCGGCC CGGGCAGCCT GGCCGCGCTG 60

CAGCGCTTTA GCCATCAGCA CATCTGGATC GTCTGCGACG GCTTCCTGGC GCGCTCGCCG 120

CTGCTTGACC GACTGCGCGC CGCGCTGCCC GCCAGCAACC GCGTCAGCGT GTTCAGCGAT 180

ATTACACCGG ATCCGACCAT TCACACCGTG GCGAAAGGGA TAGCGCAGAT GCAGGCCCTG 240

CGTCCGCAGG TGGTGATCGG CTTCGGCGGC GGCTCGGCGA TGGATGCCGC CAAGGCTATC 300

GTCTGGTTCA GCCAGCAGGG CGGTCTGCCT GTTGACACCT GCGTGGCGAT CCCCACCACC 360

AGCGGTACCG GTTCGGAAGT GACCAGCGCC TGCGTCATCA GCGACCCGGA AAAAGGGATC 420

AAGTACCCGC TGTTCCATGA GGCGCTCTGT CCCGACATGG CGATCATCGA CCCGACGCTG 480

GTGGTTAGCG TACCGCCCAC CATCACAGCC CATACCGGGC TGGACGCGCT GACCCACGCC 540

CTGGAGGCAT GGGTCTCGCC GCAGGCCACC GATTTTACCG ATGCGCTGGC GGAAAAGGCC 600

GCCAGGCTGG TGTTTCGCGC CCTGCCCGTT GCGATTCGTC AGGGCGACTG CATTGCGACC 660

CGCAGCAAAA TGCACAATGC ATCAACCCTC GCCGGTATGG CCTTTAGCCA GGCTGGCCTT 720

GGGCTCAATC ATGCGATCGC CCATCAGCTT GGCGGCCAGT TTCACCTCCC CCATGGCCTG 780

GCCAATGCGC TGCTGCTGAC CGCGGTGATC CGCTTCAATG CCGGCGAGCC GCGAGCGGCT 840

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GAGGCTTTCC AGGCGCTGCT TACCGCGGTG GAAACGCTGA AACAGCAGTG CGCCATTCCC 960

CCCCTCAAGG GCGCGCTGCA GGAAAAGTAT CCCCTTTTCT TATCGCATCA ACCAGTTCAA 1020

CATCATTGCT CAGACGCACC TGCCCGCACA GCACGAAACC GACCAGGTGG CCGGCAATCA 1080

CCAGCGGGAT GGAAAAATCG GTTAACCCCG CATGACAGCG GTAGATACAC AGCTGTCTTT 1140

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GTGCTGACGC ATCAGCTGGC AAAACGGCGT GAAATTAAAC AATTCAGAAA TCTCATCACC 1260

GTGAATATTG ACGACCACAA CCGCCAGACT GGTGGCTTGC GCAAAATCCT GTGCGATTTT 1320

ATTGATGAGT TCTGA 1335

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Ser Lys Arg Phe Glu Ala Leu Ala Lys Arg Pro Val Asn Gln
1 5 10 15

Asp Gly Phe Val Lys Glu Trp Ile Glu Glu Gly Phe Ile Ala Met Glu
20 25 30

Ser Pro Asn Asp Pro Lys Pro Ser Ile Arg Ile Val Asn Gly Ala Val
35 40 45

Thr Glu Leu Asp Gly Lys Pro Val Glu Gln Phe Asp Leu Ile Asp His
50 55 60

Phe Ile Ala Arg Tyr Gly Ile Asn Leu Ala Arg Ala Glu Glu Val Met
65 70 75 80

Ala Met Asp Ser Val Lys Leu Ala Asn Met Leu Cys Asp Pro Asn Val
85 90 95

Lys Arg Ser Asp
100

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Ser Lys Arg Phe Glu Ala Leu Ala Lys Arg Pro Val Asn Gln
1 5 10 15

Asp Gly Phe Val Lys Glu Trp Ile Glu Glu Gly Phe Ile Ala Met Glu
20 25 30

Ser Pro Asn Asp Pro Lys Pro Ser Ile Lys Ile Val Asn Gly Ala Val
35 40 45

Thr Glu Leu Asp Gly Lys Pro Val Ser Glu Phe
50 55

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 554 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Arg Ser Lys Arg Phe Glu Ala Leu Ala Lys Arg Pro Val Asn Gln
1 5 10 15

Asp Gly Phe Val Lys Glu Trp Ile Glu Glu Gly Phe Ile Ala Met Glu
20 25 30

Ser Pro Asn Asp Pro Lys Pro Ser Ile Arg Ile Val Asn Gly Ala Val
35 40 45

Thr Glu Leu Asp Gly Lys Pro Val Glu Gln Phe Asp Leu Ile Asp His
50 55 60

Phe Ile Ala Arg Tyr Gly Ile Asn Leu Ala Arg Ala Glu Glu Val Met
65 70 75 80

Ala Met Asp Ser Val Lys Leu Ala Asn Met Leu Cys Asp Pro Asn Val
85 90 95

Lys Arg Ser Asp Ile Val Pro Leu Thr Thr Ala Met Thr Pro Ala Lys
100 105 110

Ile Val Glu Val Val Ser His Met Asn Val Val Glu Met Met Met Ala
115 120 125

Met Gln Lys Met Arg Ala Arg Arg Thr Pro Ser Gln Gln Ala His Val
130 135 140

Thr Asn Ile Lys Asp Asn Pro Val Gln Ile Ala Ala Asp Ala Ala Glu
145 150 155 160

Gly Ala Trp Arg Gly Phe Asp Glu Gln Glu Thr Thr Val Ala Val Ala
165 170 175

Arg Tyr Ala Pro Phe Asn Ala Ile Ala Leu Leu Val Gly Ser Gln Val
180 185 190

Gly Arg Pro Gly Val Leu Thr Gln Cys Ser Leu Glu Glu Ala Thr Glu
195 200 205

Leu Lys Leu Gly Met Leu Gly His Thr Cys Tyr Ala Glu Thr Ile Ser
210 215 220

Val Tyr Gly Thr Glu Pro Val Phe Thr Asp Gly Asp Asp Thr Pro Trp
225 230 235 240

Ser Lys Gly Phe Leu Ala Ser Ser Tyr Ala Ser Arg Gly Leu Lys Met
245 250 255

Arg Phe Thr Ser Gly Ser Gly Ser Glu Val Gln Met Gly Tyr Ala Glu
260 265 270

Gly Lys Ser Met Leu Tyr Leu Glu Ala Arg Cys Ile Tyr Ile Thr Lys
275 280 285

Ala Ala Gly Val Gln Gly Leu Gln Asn Gly Ser Val Ser Cys Ile Gly
290 295 300

Val Pro Ser Ala Val Pro Ser Gly Ile Arg Ala Val Leu Ala Glu Asn
305 310 315 320

Leu Ile Cys Ser Ala Leu Asp Leu Glu Cys Ala Ser Ser Asn Asp Gln
325 330 335

Thr Phe Thr His Ser Asp Met Arg Arg Thr Ala Arg Leu Leu Met Gln
340 345 350

Phe Leu Pro Gly Thr Asp Phe Ile Ser Ser Gly Tyr Ser Ala Val Pro
355 360 365

Asn Tyr Asp Asn Met Phe Ala Gly Ser Asn Glu Asp Ala Glu Asp Phe
370 375 380

Asp Asp Tyr Asn Val Ile Gln Arg Asp Leu Lys Val Asp Gly Gly Leu
385 390 395 400

Arg Pro Val Arg Glu Glu Asp Val Ile Ala Ile Arg Asn Lys Ala Ala
405 410 415

Arg Ala Leu Gln Ala Val Phe Ala Gly Met Gly Leu Pro Pro Ile Thr
420 425 430

Asp Glu Glu Val Glu Ala Ala Thr Tyr Ala His Gly Ser Lys Asp Met
435 440 445

Pro Glu Arg Asn Ile Val Glu Asp Ile Lys Phe Ala Gln Glu Ile Ile
450 455 460

Asn Lys Asn Arg Asn Gly Leu Glu Val Val Lys Ala Leu Ala Lys Gly
465 470 475 480

Gly Phe Pro Asp Val Ala Gln Asp Met Leu Asn Ile Gln Lys Ala Lys
485 490 495

Leu Thr Gly Asp Tyr Leu His Thr Ser Ala Ile Ile Val Gly Glu Gly
500 505 510

Gln Val Leu Ser Ala Val Asn Asp Val Asn Asp Tyr Ala Gly Pro Ala
515 520 525

Thr Gly Tyr Arg Leu Gln Gly Glu Arg Trp Glu Glu Ile Lys Asn Ile
530 535 540

Pro Gly Ala Leu Asp Pro Asn Glu Leu Gly
545 550

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 555 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Arg Arg Ser Lys Arg Phe Glu Val Leu Ala Gln Arg Pro Val Asn
1 5 10 15

Gln Asp Gly Leu Ile Gly Glu Trp Pro Glu Glu Gly Leu Ile Ala Met
20 25 30

Glu Ser Pro Tyr Asp Pro Ala Ser Ser Val Lys Val Glu Asn Gly Arg
35 40 45

Ile Val Glu Leu Asp Gly Lys Ser Arg Ala Glu Phe Asp Met Ile Asp
50 55 60

Arg Phe Ile Ala Asp Tyr Ala Ile Asn Val Pro Glu Ala Glu Arg Ala
65 70 75 80

Met Gln Leu Asp Ala Leu Glu Ile Ala Arg Met Leu Val Asp Ile His
85 90 95

Val Ser Arg Glu Glu Ile Ile Ala Ile Thr Thr Ala Ile Thr Pro Ala
100 105 110

Lys Arg Leu Glu Val Met Ala Gln Met Asn Val Val Glu Met Met Met
115 120 125

Ala Leu Gln Lys Met Arg Ala Arg Arg Thr Pro Ser Asn Gln Cys His
130 135 140

Val Thr Asn Leu Lys Asp Asn Pro Val Gln Ile Ala Ala Asp Ala Ala
145 150 155 160

Glu Ala Gly Ile Arg Gly Phe Ser Glu Gln Glu Thr Thr Val Gly Ile
165 170 175

Ala Arg Tyr Ala Pro Phe Asn Ala Leu Ala Leu Leu Val Gly Ser Gln
180 185 190

Cys Gly Ala Pro Gly Val Leu Thr Gln Cys Ser Val Glu Glu Ala Thr
195 200 205

Glu Leu Glu Leu Gly Met Arg Gly Leu Thr Ser Tyr Ala Glu Thr Val
210 215 220

Ser Val Tyr Gly Thr Glu Ser Val Phe Thr Asp Gly Asp Asp Thr Pro
225 230 235 240

Trp Ser Lys Ala Phe Leu Ala Ser Ala Tyr Ala Ser Arg Gly Leu Lys
245 250 255

Met Arg Tyr Thr Ser Gly Thr Gly Ser Glu Ala Leu Met Gly Tyr Ser
260 265 270

Glu Ser Lys Ser Met Leu Tyr Leu Glu Ser Arg Cys Ile Phe Ile Thr
275 280 285

Lys Gly Ala Gly Val Gln Gly Leu Gln Asn Gly Ala Val Ser Cys Ile
290 295 300

Gly Met Thr Gly Ala Val Pro Ser Gly Ile Arg Ala Val Leu Ala Glu
305 310 315 320

Asn Leu Ile Ala Ser Met Leu Asp Leu Glu Val Ala Ser Ala Asn Asp
325 330 335

Gln Thr Phe Ser His Ser Asp Ile Arg Arg Thr Ala Arg Thr Leu Met
340 345 350

Gln Met Leu Pro Gly Thr Asp Phe Ile Phe Ser Gly Tyr Ser Ala Val
355 360 365

Pro Asn Tyr Asp Asn Met Phe Ala Gly Ser Asn Phe Asp Ala Glu Asp
370 375 380

Phe Asp Asp Tyr Asn Ile Leu Gln Arg Asp Leu Met Val Asp Gly Gly
385 390 395 400

Leu Arg Pro Val Thr Glu Glu Glu Thr Ile Ala Ile Arg Asn Lys Ala
405 410 415

Ala Arg Ala Ile Gln Ala Val Phe Arg Glu Leu Gly Leu Pro Leu Ile
420 425 430

Ser Asp Glu Glu Val Asp Ala Ala Thr Tyr Ala His Gly Ser Lys Asp
435 440 445

Met Pro Ala Arg Asn Val Val Glu Asp Leu Ala Ala Val Glu Glu Met
450 455 460

Met Lys Arg Asn Ile Thr Gly Leu Asp Ile Val Gly Ala Leu Ser Ser
465 470 475 480

```
Ser Gly Phe Glu Asp Ile Ala Ser Asn Ile Leu Asn Met Leu Arg Gln
                485                 490                 495

Arg Val Thr Gly Asp Tyr Leu Gln Thr Ser Ala Ile Leu Asp Arg Gln
            500                 505                 510

Phe Asp Val Val Ser Ala Val Asn Asp Ile Asn Asp Tyr Gln Gly Pro
        515                 520                 525

Gly Thr Gly Tyr Arg Ile Ser Ala Glu Arg Trp Ala Glu Ile Lys Asn
    530                 535                 540

Ile Ala Gly Val Val Gln Pro Gly Ser Ile Glu
545                 550                 555
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 131 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Arg Ala Val Leu Ala Glu Asn Leu Ile Ala Ser Met Leu Asp Leu Glu
1               5                  10                  15

Val Ala Ser Ala Asn Asp Gln Thr Phe Ser His Ser Asp Ile Arg Arg
            20                  25                  30

Thr Ala Arg Thr Leu Met Gln Met Leu Pro Gly Thr Asp Phe Ile Phe
        35                  40                  45

Ser Gly Tyr Ser Ala Val Pro Asn Tyr Asp Asn Met Phe Ala Gly Ser
    50                  55                  60

Asn Phe Asp Ala Glu Asp Phe Asp Asp Tyr Asn Ile Leu Gln Arg Asp
65                  70                  75                  80

Leu Met Val Asp Gly Gly Leu Arg Pro Val Thr Glu Ala Glu Thr Ile
                85                  90                  95

Ala Ile Arg Gln Lys Ala Ala Arg Ala Ile Gln Ala Val Phe Arg Glu
            100                 105                 110

Leu Gly Leu Pro Pro Ile Ala Asp Glu Glu Val Glu Ala Ala Thr Tyr
        115                 120                 125

Ala Gln Gly
    130
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 150 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Val Ser Cys Ile Gly Met Thr Gly Ala Val Pro Ser Gly Ile Arg Ala
1               5                  10                  15

Val Leu Ala Glu Asn Leu Ile Ala Ser Met Leu Asp Leu Glu Val Ala
            20                  25                  30

Ser Ala Asn Asp Gln Thr Phe Ser His Ser Asp Ile Arg Arg Thr Ala
        35                  40                  45

Arg Thr Leu Met Gln Met Leu Pro Gly Thr Asp Phe Ile Phe Ser Gly
    50                  55                  60

Tyr Ser Ala Val Pro Asn Tyr Asp Asn Met Phe Ala Gly Ser Asn Phe
65                  70                  75                  80

Asp Ala Glu Asp Phe Asp Asp Tyr Asn Ile Leu Gln Arg Asp Leu Met
                85                  90                  95

Val Asp Gly Gly Leu Arg Pro Val Thr Glu Glu Glu Thr Ile Ala Ile
            100                 105                 110

Arg Asn Lys Ala Ala Arg Ala Ile Gln Ala Val Phe Arg Glu Leu Gly
        115                 120                 125

Leu Pro Leu Ile Ser Asp Glu Glu Val Asp Ala Ala Thr Tyr Ala His
    130                 135                 140

Gly Ser Lys Asp Met Pro
145                 150
```

WHAT IS CLAIMED IS:

1. A cosmid comprising a DNA fragment of about 35 kb isolated from *Klebsiella pneumoniae* wherein said fragment encodes an active diol dehydratase enzyme having the restriction digest in Figure 5, columns numbered 4.

2. A transformed microorganism comprising a host microorganism and the cosmid of Claim 1.

3. The transformed microorganism of Claim 2 wherein the host microorganism is *E. coli*, and which is deposited with the American Type Culture Collection as accession number ATCC 69790.

4. The cosmid of Claim 1 which when transformed into bacteria causes metabolism of glycerol to 1,3-propanediol.

5. A transformed microorganism comprising a host microorganism and a DNA fragment of the cosmid of Claim 1, said fragment encoding an active functional protein.

6. A DNA fragment comprising a gene encoding a diol dehydratase enzyme, said gene encompassed by the cosmid of Claim 1.

7. A gene encoding an active diol dehydratase enzyme having the DNA sequence as listed in SEQ ID NO:1.

8. A gene encoding an active alcohol dehydrogenase having the DNA sequence as listed in SEQ ID NO:2.

9. A transformed microorganism comprising a host microorganism and the DNA sequence of Claim 7 or Claim 8.

10. A transformed microorganism comprising *E. coli* DH5α and the DNA sequence of Claim 7 or Claim 8.

11. A process comprising the bioconversion of a carbon substrate by transforming a microbial host with genes capable of expressing a diol dehydratase and contacting said transformed host with said substrate.

12. A process comprising the bioconversion of a carbon substrate by transforming a microbial host with genes derived from a cosmid comprising a fragment of about 35 kb isolated from *Klebsiella pneumoniae* wherein said genes encode an active diol dehydratase enzyme and any other functional bacterial protein encoded by said cosmid, and contacting said transformed host with said substrate.

13. The process of Claim 12 wherein said other functional bacterial protein is an alcohol dehydrogenase.

14. The process of Claim 11 or 12 wherein the carbon substrate is selected from the group consisting of ethyleneglycol, 1,2-propanediol, glycerol and 2,3-butanediol.

15. The process of Claim 14 wherein the carbon substrate is glycerol.

16. The process of Claim 15 wherein the glycerol is converted to 1,3-propanediol.

17. The process of Claim 11 or 12 wherein the microbial host is selected from the group consisting of members of the genera *Escherichia*, *Bacillus*, *Klebsiella*, *Citrobacter*, *Saccharomyces*, *Clostridium* and *Pichia*.

18. The process of Claim 17 wherein the microbial host is selected from the group consisting of members of species *E. coli*, *Bacillus subtilis*, *Bacillus licheniformis* and *Pichia pastoris*.

19. The process of Claim 18 wherein the microbial host is *E. coli*.

20. The process of Claim 11 or 12 wherein the genes are diol dehydratase genes isolated from the group consisting of members of the genera *Klebsiella* sp., *Clostridia* sp., *Salmonella* sp. and *Citrobacter* sp.

21. The process of Claim 16 wherein said transformed host is recombinant *E. coli* DH5α containing a gene encoding diol dehydratase enzyme

wherein said gene comprises the DNA sequence of SEQ ID NO:1.

22. The process of Claim 20 wherein said transformed host is recombinant *E. coli* DH5α containing a gene encoding diol dehydratase enzyme wherein said gene comprises the DNA sequence of SEQ ID NO:1.

23. The product of the process of Claim 11 or 12.


pduC
pduB
pduA
pduF
pocR

FIGURE 2

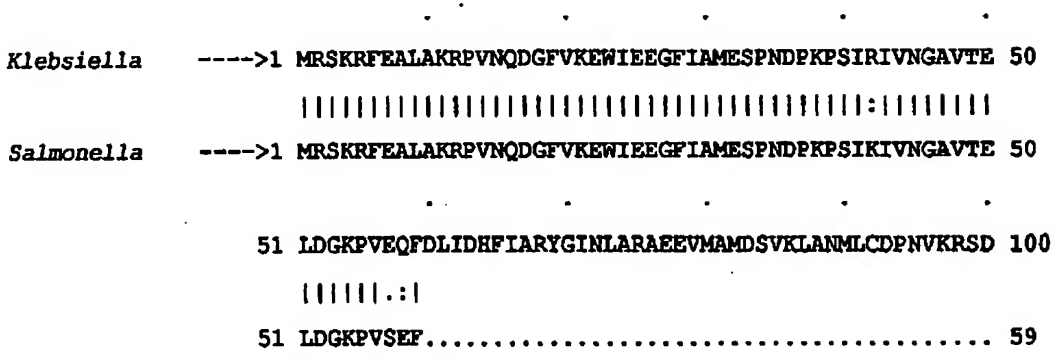
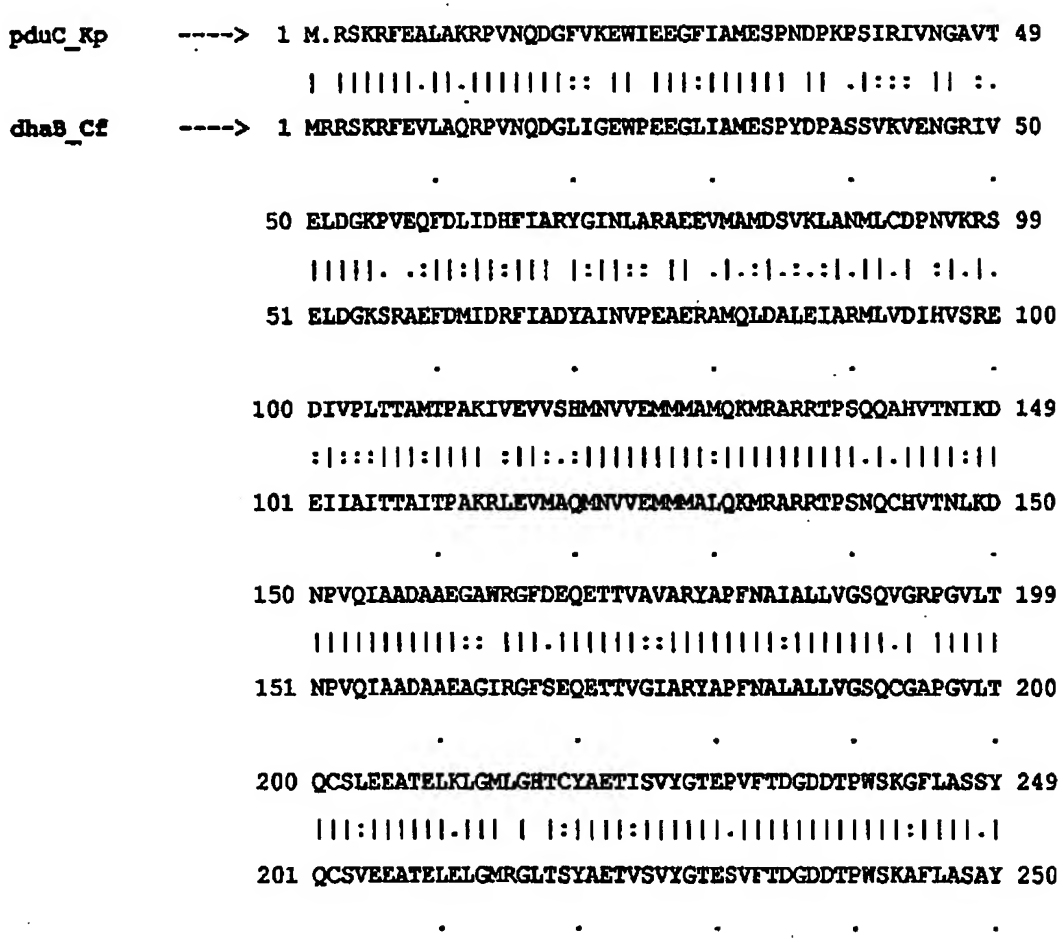

Klebsiella ---->1 MRSKRFEALAKRPVNQDGFVKEWIEEGFIAMESPNDPKPSIRIVNGAVTE 50
Salmonella ---->1 MRSKRFEALAKRPVNQDGFVKEWIEEGFIAMESPNDPKPSIKIVNGAVTE 50
51 LDGKPVEQFDLIDHFIARYGINLARAEEVMAMDSVKLANMLCDPNVKRSD 100
51 LDGKPVSEF..... 59

FIGURE 3


pduC_Kp ----> 1 M.RSKRFEALAKRPVNQDGFVKEWIEEGFIAMESPNDPKPSIRIVNGAVT 49
dhaB_Cf ----> 1 MRRSKRFEVLAQRPVNQDGLIGEWPEEGLIAMESPYDPASSVKVENGRIV 50
50 ELDGKPVEQFDLIDHFIARYGINLARAEEVMAMDSVKLANMLCDPNVKRS 99
51 ELDGKSRAEFDMIDRFIADYAINVPEAERAMQLDALEIARMLVDIHVSRE 100
100 DIVPLTTAMTPAKIVEVVSHMNVVEMMMAMQKMRARRTPSQQAHVTNIKD 149
101 EIIAITTAITPAKRLEVMAQMNVVEMMMALQKMRARRTPSNQCHVTNLKD 150
150 NPVQIAADAAEGAWRGFDEQETTVAVARYAPFNAIALLVGSQVGRPGVLT 199
151 NPVQIAADAAEAGIRGFSEQETTVGIARYAPFNALALLVGSQCGAPGVLT 200
200 QCSLEEATELKLGMLGHTCYAETISVYGTEPVFTDGDDTPWSKGFLASSY 249
201 QCSVEEATELELGMRGLTSYAETVSVYGTESVFTDGDDTPWSKAFLASAY 250

```
250 ASRGLKMRFTSGGSEVQMGYAEGKSMLYLEARCIYITKAAGVQGLQNGS 299
    ||||||||:|||-|||- |||-|:|||||||-|||:-|||:|||||||-|-
251 ASRGLRMRYTSGTGSEALMGYSESKSMLYLESRCIFITKGAGVQGLQNGA 300
    .         .         .         .         .         .
300 VSCIGVFSAVFSGIRAVLAENLICSALDLECASSNDQTFTHSDMRRTARL 349
    |||||:..:|||||||||||||||||-| ||||-||-|||||-|||:|||||
301 VSCIGMTGAVFSGIRAVLAENLIASMLDLEVASANDQTFSHSDIRRTART 350
    .         .         .         .         .         .
350 LMQFLPGTDFISSGYSAVFNYDNMFAGSNEDAEDFTDDYNVIQRDLKVDGG 399
    |||:|||||||| ||||||||||||||||||| |||||||||:::||||-||||
351 LMQMLPGTDFIFSGYSAVFNTDNMFAGSNFDAEDFTDDYNILQRDLMVDGG 400
    .         .         .         .         .         .
400 LRPVREEDVIAIRNKAARALQAVFAGMGLPPITDEVEAATYAHGSKDMP 449
    |||| ||:-.|||||||||||:|||| ::|||| |-|||||:|||||||||||||
401 LRPVTEETIAIRNKAARATQAVFRELGLPLISDEEVDAATYAHGSKDMP 450
    .         .         .         .         .         .
450 ERNIVEDIKFAQEIINKNRNGLEVVKALAKGGFPDVAQDMLNTQKALTG 499
    .||:|||:  .:|:::-:| .||::| ||..:||.-|:| ::||: :.::||
451 ARNVVEDLAAVEEMMKRNITGLDIVGALSSSGFEDIASNIIAMMLRQKVTG 500
    .         .         .         .         .         .
500 DYLHTSAIIVGEGQVLSAVNDVNDYAGPATGYRLQGERMEEIKNTPGALD 549
    |||:|||||:  : :|:||||||:|||-||-|||||: :|||-|||||:|-::
501 DYLQTSAILDRQFDVVSAVNDINDYQGPGTGYRISAERWAEIKNIAGVVQ 550
```

550 PNELG* 555
|...::
551 PGSIE* 556

FIGURE 4

```
                                    .         .         .         .         .
dhaB_Kp.--->    1 ..............RAVLAENLIASMLDLEVASANDQTFSHSDIRRTART 36
                                ||||||||||||||||||||||||||||||||||||
dhaB_Cf.--->  301 VSCIGMTGAVPSGIRAVLAENLIASMLDLEVASANDQTFSHSDIRRTART 350

                            .         .         .         .         .
               37 LMQMLPGTDFIFSGYSAVPNYDNMFAGSNFDAEDFDDYNILQRDLMVDGG 86
                  ||||||||||||||||||||||||||||||||||||||||||||||||||
              351 LMQMLPGTDFIFSGYSAVPNYDNMFAGSNFDAEDFDDYNILQRDLMVDGG 400

                            .         .         .         .         .
               87 LRPVTEAETIAIRQKAARAIQAVFRELGLPPIADEEVEAATYAQG..... 131
                  ||||||.||||||.|||||||||||||||| |.||||:|||||:|
              401 LRPVTEEETIAIRNKAARAIQAVFRELGLPLISDEEVDAATYAHGSKDMP 450
```

International Application No: PCT/ 96/ 06163

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 6, line 34-35 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit [5]	Accession Number [6]
18 April 1995 (18.04.95)	69789

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

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was

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PCT/US 96/06163

International Application No ~~PCT/~~

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 6, line 34-35 ~~of the description~~

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
18 April 1995 (18.04.95)	69790

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

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(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

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INTERNATIONAL SEARCH REPORT

Internatior pplication No
PCT/US 96/06163

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/60 C12N15/53 C12P7/18 C12N9/04 C12N9/88
C12N15/74 C12N15/79 C12N1/21 C12N1/19

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 118, no. 7, 15 February 1993 Columbus, Ohio, US; abstract no. 56047, DANIEL, ROLF ET AL: "Growth temperature-dependent activity of glycerol dehydratase in Escherichia coli expressing the Citrobacter freundii dha regulon" XP002010809 see abstract & FEMS MICROBIOL. LETT. (1992), 100(1-3), 281-5 CODEN: FMLED7;ISSN: 0378-1097, --- -/--	1,11

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 August 1996	0 3. 09. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Delanghe, L

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INTERNATIONAL SEARCH REPORT

Internatio: .pplication No
PCT/US 96/06163

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 116, no. 9, 2 March 1992 Columbus, Ohio, US; abstract no. 82153, TONG, I TEH ET AL: "1,3- Propanediol production by Escherichia coli expressing genes from the Klebsiella pneumoniae dha regulon" XP002010810 see abstract & APPL. ENVIRON. MICROBIOL. (1991), 57(12), 3541-6 CODEN: AEMIDF;ISSN: 0099-2240, ---	1,8,11
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 13, 31 March 1995, MD US, pages 7142-7148, XP002011582 TAKAMASA TOBIMATSU ET AL.: "Molecular cloning,sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of Klebsiella oxytoca."	1
X	see the whole document ---	11, 17-20,23
A	J. BACTERIOL. (1995), 177(15), 4392-401 CODEN: JOBAAY;ISSN: 0021-9193, XP002011583 DANIEL, ROLF ET AL: "Biochemical and molecular characterization of the oxidative branch of glycerol utilization by Citrobacter freundii" see the whole document ---	8,13
A	J. BACTERIOL. (1992), 174(7), 2253-66 CODEN: JOBAAY;ISSN: 0021-9193, XP002011584 BOBIK, THOMAS A. ET AL: "A single regulatory gene integrates control of vitamin B12 synthesis and propanediol degradation" see the whole document ---	1,8,11
A	CHEMICAL ABSTRACTS, vol. 111, no. 5, 31 July 1989 Columbus, Ohio, US; abstract no. 36402, SPRENGER, G. A. ET AL: "Anaerobic growth of Escherichia coli on glycerol by importing genes of the dha regulon from Klebsiella pneumoniae" XP002011587 see abstract & J. GEN. MICROBIOL. (1989), 135(5), 1255-62 CODEN: JGMIAN;ISSN: 0022-1287, --- -/--	1

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US 96/06163

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 96, no. 21, 24 May 1982 Columbus, Ohio, US; abstract no. 177658, FORAGE, ROBERT G. ET AL: "Glycerol fermentation in Klebsiella pneumoniae: functions of the coenzyme B12-dependent glycerol and diol dehydratases" XP002011588 see abstract & J. BACTERIOL. (1982), 149(2), 413-19 CODEN: JOBAAY;ISSN: 0021-9193, ---	1,8,11
A	J.BACTERIOL, vol. 177, no. 8, 1995, pages 2151-2156, XP002011585 DANIEL,ROLF ET AL.: "Purification of 1,3-propanediol dehydrogenase from Citrobacter freundii and cloning,sequencing and overexpression of the corresponding gene in Escherichia coli. " see the whole document ---	8,13
A	GENE, vol. 85, 1989, AMSTERDAM NL, pages 209-214, XP002011586 PAIGE E. GOODLOVE ET AL.: "Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of Escherichia coli" see the whole document -----	8

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